HYOSCYAMINE 6 b-HYDROXYLASE GENE ISOLATION FROM *IN VITRO* CULTURED ROOTS OF *HYOSCYAMUS NIGER* L. AND *HYOSCAYMUS TENUICAULIS* SCHONBECK-TEMESY

HASSAN EBRAHIMZADEH, AFSANEH TEIMOORI, TAHMINEH LOHRASEBI

Department of Biology, Faculty of Science, University of Tehran

ABSTRACT

The H6H gene for hyoscyamine 6β-hydroxylase, which converts hyoscyamine to scopolamine, was isolated from *H. niger* and *H. tenuicaulis*. The roots of 14 days sterile seedlings were transferred to a modified liquid B_5 medium containing 1µM indolebutyric acid, and after appearance of the lateral roots, subcultured in a free hormone medium. Following a week, the total cellular RNA of the roots was extracted. cDNA of H6H gene was synthetized by RT-PCR, favored gene was amplified by the PCR.

Keywords: *Hyoscyamus niger, Hyoscyamus tenuicaulis,*Cultured roots, H6H gene, cDNA synthesis.

INTRODUCTION

Hyoscyamine and scopolamine are the most common tropane alkaloids of the *Solanaceae* and have been used for their medicinal properties (1). Hyoscyamine 6β-hydroxylase (H6H) catalyzes the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine, as well as its epoxidation to scopolamine (2). RNA blotting hybrizidation studies, detected considerable amounts of H6H mRNA in cultured roots, but not in leaves, stems and cell cultures of H*yoscyamus niger* (3). Immunohisto-chemical studies by monoclonal antibodies have shown that the localization of enzyme synthesis is the pericycle of root (4). Also *in situ* hybrizidation, immunohisto-chemistry and promoter GUS transgene analyses have shown that *Atropa belladonna* H6H is expressed specifically in root pericycle, tapetum and pollen mother cells (1). The enzyme has an average Mr of 40000 as determined by gel filtration on superose 12 (5). Also it has been reported cultured roots contained much more abundant H6H mRNA than plant roots (6). In this study, the H6H gene was isolated from *in vitro* cultured roots of *H. nigre* and *H. tenuicaulis*.

MATERIALS AND METHODS

Chemicals

The indolebutyric acid (IBA), ethidium bromide, macro-and micro-elements, ferrous–EDTA and vitamins of culture media were purchased from Merck Chemical Co., Ltd (Germany), agarose, DNA molecular weight marker XVII, cDNA synthesis kit, guanidinium isothiocyanate were from Boechringre and Mannhaim Cheimical Co., Ltd (Germany). The two primers were obtained from Roch Chemical Co., Ltd (Germany).

Apparatuses

Spectrophotometer: Shimadzu UV visible recording spectrophotometer UV-160 model (Japan), PCR: Omnigene (Hybaid) model (England), Electrophoresis: Paya Pazhohesh model (Iran).

Plant materials

Seeds of *Hyoscyamus niger and Hyoscyamus tenuicaulis* were collected from Kandowan region (the North of Iran, 2400 meters altitude) and Busheher (South of Iran) respectively.After sterilization (7), the seeds of *H. niger* were kept for 48 hours at -20°C to make rapid germination. The seeds of *H. tenucaulis* didn't require any cold treatment, since its germination was very rapid (after 2-3 days). Roots (from 14 days sterile seedlings) were transferred to modified liquid B_5 medium (macro- and micro-elements of B_5 medium, ferrous EDTA and vitamin of MS medium), containing 1µM indolebutyric acid and 3% sucrose and maintained on a rotary shakers at

Correspondence: Dr. H. Ebrahimzadeh, Department of Biology, Faculty of Science, University of Tehran, Tehran, 14155-6455, IRAN. Email: ebizadeh@khayam.ut.ac.ir

100 rpm in the dark at 25°C (2,8). Following appearance of the lateral roots, samples were transferrd to a solid modified B_5 medium free hormone, and after a week were used for extraction of total cellular RNA (Figure 1).

Total cellular RNA extraction

The total RNA extraction was performed and compared by two methods, A: by GITC: phenol method (REX™ procedure) (9), B: by cesium chloride gradient method (10a).

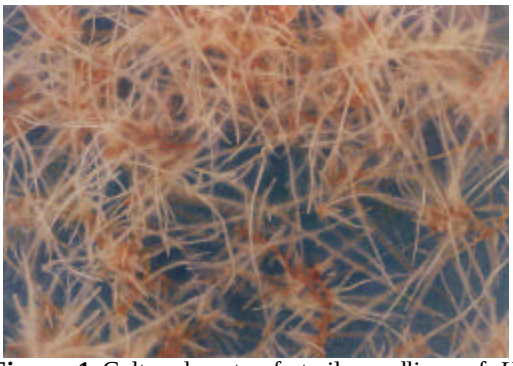

Figure 1 Cultured roots of sterile seedlings of *H. tenuicaulis* that were used for total RNA extraction.

Electrophoresis of total cellular RNA

Before using RNA preparation in either, cDNA synthesis or blotting techniques, the accuracy of isolated RNA should be established (by detection of RNA bands on an agarose gel). The best concentration of agarose gel for RNA electrophoresis is 1.5 percent. The voltage applied across the gel was 40V. Staining was performed by ethidium bromide (0.5µg/ml). Five µg of total RNA was loaded in each well. The presence of two ribosomal RNA bands (28S and 18S) and small smear between them, proved the precision of the RNA accuracy (also little DNA contamination) (10_b).

cDNA synthesis

The buffer No. I for the first strand synthesis (4µl), RNase inhibitor (1µl), dNTP mix (10mM-2µl), oligo $(dT)_{15}$ primer (2µ), total RNA (4µg), AMV reverse trancriptase (2µl) and dH_2O up to 20µl were pipetted into a sterile microfuge tube on ice and were incubated for 60 min at 42°C.Then the buffer No. II for the second strand synthesis (540 µl), RNase H (1µl), E.coli DNA polymerase I (5µl) and dH_2O up to 100µl were pipetted into the same microfuge tube and were incubated for 60 min at 12°C, 60 min at 22°C and 10 min at 65°C. Then 4µl DNA polymerase were added to the same tube, and the contents incubated for 10 min at 37°C. At the end, in order to stop the reaction, 10µl EDTA (0.2M-pH: 7.2) and 2µl of Sarkosyl solution 10% were added (11).

Polymerase chain reaction

PCR was achieved by using taq polymerase, 5µl of RT-PCR product, and two primers that would anneal at the start of the H6H gene. The reaction conditions were : 95°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute (25 cycle) (12).

Primer 1:
GAGGATCCATTTGATGGCTAGTTTTGTG (28mer)

Primer 2:
TGCCTTAAGTTAGACATTGATTTTATATGG (30mer)

Enzymatic digestion with PVUII

Restriction endonuclease PVUII recognizes the sequence of CAG/CTG and generates fragments with blunt ends. In a sterile microfuge tube were pipetted, RT-PCR product (2µl), PVU II (2.5 unit), SURE/cut buffer M 10x (2µl) and dH_2O was added to the tube up to 20µl. The tube was incubated at 37°C for 30 min, and the electrophoresis was performed on a 1.5% agarose gel (10c).

Electrophoresis of PCR and enzymatic digestion products

The correct amplification of the H6H gene and its enzymatic digestion were detected by electrophoresis on a 1.5% agarose gel. One µg of DNA size marker, 5-10 µl of PCR product and 3µl of enzymatic digestion product were loaded in the wells. The staining was performed by ethidium bromide (0.5µg/ml) (10d).

RESULTS AND DISCUSSION

The isolation and characterization of mRNA are the most important part of the gene expression studies of an organism (13). In this study, total cellular RNA was extracted by two methods (guanidinium thiocyanate (4M) phenol chloroform method and cesium chloride gradient). The results showed that in spite of appearance of RNA circle in the middle of centrifuge tube, by cesium chloride gradient,

the final RNA preparation wasn't completely free of DNA, but its DNA contamination was considerably lower than phenol–chloroform method (Figure 2).

Figure 2. Electrophoresis of total cellular RNA on a 1.5% agarose gel. Arrows represent 28S and 18S rRNA bands.

It has been reported that phenol extraction method is a complex procedure which can cause aggregation of RNA, removal of poly (A) regions from mRNA end for the RNA extraction and for this reasons sucrose gradient, oligo dT-cellulose or poly(U)-Sepharose column are better methods (13).

Our results are not in accordance with this report. Experiments well showed that lower steps in nucleic acid extraction (especially RNA), caused final preparation to have more accurate nucleic acid. In 1990, a rapid, reliable and quantitative method for undegraded RNA was reported (9). Our results correlate well with this report. Cesium chloride method has some difficulties: because it is expensive, and requires, an ultracentrifuge (37800 rpm- for 24 hours). However, RNA accuracy wasn't very different in two methods. After each extraction, the spectrophotometric spectrum of nucleic acid preparation, at a range of 200-300 nm was determined (14).

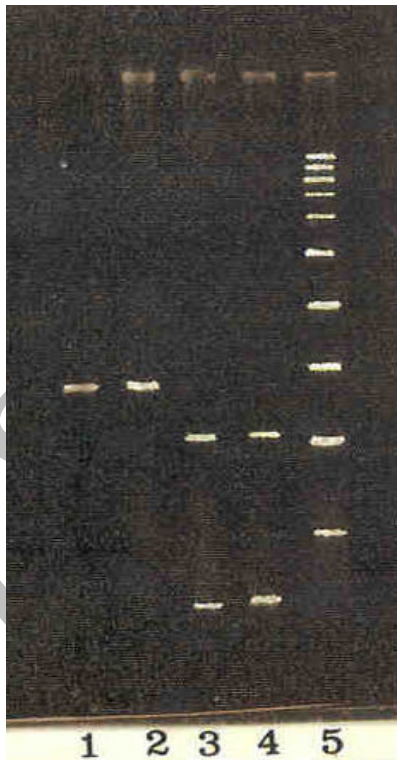

Figure 3. Electrophoresis of PCR enzymatic digestion products.
Lane 1: H6H gene from *H. niger*, Lane 2: H6H gene from *H. tenuicaulis*, Lane 3: fragments of H6H gene digested with PVUII from *H.niger*, Lane 4: fragments of H6H gene digested with PVUII from *H. tenuicaulis*, Lane 5: DNA size marker (contained 10 bands, at a range of 500-5000 bp bands).

With respect to 1 unit absorption equal to 40 µg/ml RNA, the concentration of RNA preparation was determined exactly. The ratio of 260 nm to 280 nm absorption shows the RNA accuracy (the ratios of 1.9-2 is ideal).

By using a cDNA synthesis kit and oligo (dT) primer, the H6H cDNA was synthesized from total RNA isolated from cultured roots (Oligo (dT) could be isolated from total RNA by annealing the 3' end of mRNA) (11). Then, to amplify the favored gene, RT-PCR products were used as the templates for PCR (12). The gene specific primers were designed on the basis of DNA sequence of H6H (4), to anneal the start site of the gene. In order to demonstrate

the correct amplification of H6H gene, PCR products were digested with PVUII, followed by electrophoresis on a 1.5% agarose gel. The H6H cDNA was expected to be 14000 bp long and cleavable into 1076 bp and 274 bp fragments at a PVUII site (figure 3).The results for *H. niger* and *H.tenuicaulis* were similar, and confirmed other reported results (5,8).

REFERENCES

1. Suzuki K., Yun D., Chen X., Yamada Y. and Hashimoto T. (1991). An *Atropa belladonna* hyoscyamine 6β-hydroxylase gene is differentially expressed in the root pericycle and anthers. Plant Molecular Biology, 40: 141-152.
2. Hashimoto T., Yun D. J. and Yamada Y. (1993). Purification of tropane alkaloid in genetically engineered root cultures, Phytochemistry, 32(3): 713-718.
3. Hashimoto T., Hayashi A., Amano Y., Kohono J., Jwanari H., Usuda S. and Yamada Y. (1991). Hyoscyamine 6β-hydroxylase, an enzyme involved in tropane alkaloid biosynthesis, is localized at the pericycle of the root. The Journal of Biological Chemistry, 266(7): 4648-4653.
4. Kanegae T., Kajiya H., Amano Y. Hashimoto T. and Yamada Y.(1994). Species dependent expression of the hyoscyamine 6β-hydroxylase gene in the pericycle. Plant Physiol., 105(2): 483-490.
5. Hashimoto T. and Yamada Y. (1987). Purification and Characterization of hyoscyamine 6β-hydroxylase from root cultures of *Hyoscyamus niger* L.J. Eur. Biochemistry, 164: 277-285.
6. Matsuda J., Okabe S., Hashimoto T., and Yamada Y. (1991). Molecular cloning of Hyoscyamine 6β-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, from cultured roots of *Hyoscyamus niger*. The Journal of Biological Chemistry, 266(15): 9460-9464.
7. Lohrasebi T. (1995). Genetic transformation of *Hyoscyamus tenuicaulis* by *Agrobacterium tumefaciens* and induction of hairy roots in *H.niger* by *A. rhizogenes*. MS thesis, Department of Biology, Faculty of Science, Tehran University.
8. Hashimoto T. and Yamada Y. (1986). Hyoscyamine 6β-hydroxylase, a 2-oxoglutarate – dependent dixoygenase, in alkaloid – producing root cultures. Plant Physiology, 81: 619-625.
9. Meltzer S. J., Mane S. M., Wood P. K., Johnson L. and Needelman S. W. (1990). An improvement of the single – step method of RNA isolation by acid guanidinium thiocyanate – phenol – chloroform extraction. Biotechniques, 8(2): 148-149.
10. Sambrook J., Frisch E. F. and Maniatis T. (1989). Molecular cloning (second edition), Vol 1, Cold Spring Harbor Laboratory Press, New York, (a): chapter 7 pp 19-22, (b): chapter 7 pp 31-32, (c): chapter 8 pp 60-67, (d): chapter 6 pp 3-6.
11. cDNA synthesis kit procedure from Boehringer and Mannham.
12. Hashimoto T., Yun D. J. and Yamada Y. (1993). Purification of tropane alkaloids in genetically engineered roots cultures. Phytochemistry, 32 (3): 713-718.
13. Krystosek A., Cawthon L. and Kabat D. (1975). Improved methods for purification and assay of eukaryotic messenger ribonucleic acids and ribosomes. The Journal of Biological Chemistry, 250(15): 6077-6084.
14. Robert E. and Farrell Jr. (1998). RNA methodologies (Second edition). pp: 533. Academic Press.